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Four year immunogenicity of the RTS,S/AS02$_A$ malaria vaccine in Mozambican children during a phase IIb trial

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ABSTRACT

Previous studies with the malaria vaccine RTS,S/AS02$_A$ in young children in a malaria endemic area of Mozambique have shown it to have a promising safety profile and to reduce the risk of *Plasmodium falciparum* infection and disease.

In this study, we assessed the antibody responses to the *P. falciparum* and hepatitis B components of the RTS,S/AS02$_A$ vaccine over a 45 months surveillance period in a large phase IIb trial which included 2022 children aged 1–4 years at recruitment.

The RTS,S/AS02$_A$ vaccine induced high anti-circumsporozoite antibody levels with at least 96% of children remaining seropositive during the entire follow-up period. IgG titers decayed over the first 6 months of follow-up to about 25% of the initial level, but still remained 30-fold higher until month 45 compared to controls. Children with higher levels of naturally acquired immunity at baseline, assessed by blood stage indirect fluorescent antibody test, had slightly higher anti-circumsporozoite levels, after adjusting for the effect of age.

The RTS,S/AS02$_A$ vaccine also induced high levels of anti-hepatitis B surface antigen antibodies (seroprotection >97%).

RTS,S/AS02$_A$ vaccine is immunogenic and induces long-lasting anti-circumsporozoite antibodies, persisting at least 42 months after immunization. These antibodies may play a role in protection against malaria.

1. Introduction

Plasmodium falciparum is responsible for the high malaria morbidity and mortality in malaria endemic countries, accounting for around 250 million clinical malaria cases and 863,000 deaths every year [1]. The GlaxoSmithKline (GSK) Biologicals pre-erythrocytic RTS,S malaria vaccine antigen is a virus-like particle containing a mixture of RTS, a chimeric recombinant protein combining polypeptide regions of *P. falciparum* circumsporozoite protein (CSP) and the hepatitis B virus surface antigen (HBsAg), and S, the recombinant HBsAg alone. It is formulated in the AS02 adjuvant system [2,3]. Developments of this vaccine has included sequential steps of phase I and phase IIa studies in adults in the USA [3], phase I/IIb studies in adults in The Gambia [4], and finally children and infant studies in Mozambique [5–7] and Tanzania [8]. We have shown that the vaccine is immunogenic, inducing immunoglobulin G (IgG) humoral antibodies and CD4$^+$ T cell and cytokine responses to *P. falciparum* CSP [9]. Some previous trials have shown an association between anti-CSP IgG levels in serum (measured by a standardized ELISA) and vaccine efficacy against malaria infection, but not against clinical disease [7,10,11]. The protective immune mechanism of RTS,S/AS remains poorly understood, and

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is thought to involve humoral as well as cell-mediated immunity [9].

The hepatitis B virus (HBV) HBsAg portion of RTS,S is also highly immunogenic. The quality of the immune response to vaccination with HBsAg (whether in *Engerix-B*™ or RTS,S/AS02$_A$) cannot be evaluated directly by a neutralisation test, since the hepatitis B virus does not replicate *in vitro*. Nevertheless, the generation of monoclonal antibodies against various epitopes of HBsAg has allowed the identification of a protective epitope in non human primates. The RF1 monoclonal antibody (RF1 mAb) recognises this conformational epitope on peptide 124–137 of the S protein and is able to protect chimpanzees against infection with the virus [12]. In the absence of a neutralisation assay for HBV, evaluation of the presence of RF1-like antibodies in serum from vaccinated subjects can be used as a surrogate marker of protective capacity and thus provide a qualitative evaluation of the immune response to vaccination [12].

Previous reports of the pivotal proof-of-concept trial of RTS,S/AS02$_A$ in Mozambique have presented limited immunogenicity data [5,6,13]. In this article we report a more detailed analysis of the anti-CSP, and anti-HBsAg antibody responses during the entire 45 month follow-up period of the largest phase IIb trial of RTS,S/AS conducted.

2. Materials and methods

2.1. Study site

This study was conducted by the Centro de Investigação em Saúde de Manhiça, located in the Manhiça District, Southern Mozambique, from April 2003 to May 2007. The characteristics of the area have been described in detail elsewhere [14–16]. Hepatitis B immunization (given at 2, 3 and 4 months of age together with DTPw and oral Polio vaccines) was introduced in the Expanded Program on Immunization (EPI) in Mozambique in July 2001.

2.2. Study design

This study was a phase IIb double-blind, randomised controlled trial to assess the efficacy, safety and immunogenicity of the candidate RTS,S/AS02$_A$ malaria vaccine RTS,S/AS02$_A$. Details of the candidate malaria vaccine, the control vaccines, the trial design and efficacy and safety analyses for the double-blind (study months 0–8.5), single-blind (study months 8.5–21) and open phases (study months 21–45) have been presented elsewhere [5,6,13,17]. Briefly, 2022 healthy children aged 1–4 years were enrolled to receive either the candidate malaria vaccine or a comparator vaccine after written or thumb printed informed consent provided by their parents/guardians. HBsAg status was assessed at baseline, but positivity was not an exclusion criterion for the trial.

The RTS,S/AS02$_A$ and control vaccines were administered intramuscularly in the deltoid following a 0, 1, 2-month schedule. Children in the control group aged 24 months and older received three paediatric doses (0.5 ml) of hepatitis B vaccine (*Engerix-B*™, GSK Biologicals, Rixensart, Belgium). Children under 24 months in the control group had already received hepatitis B immunization by the time they were enrolled in the trial as part of their previous EPI immunization, and were therefore vaccinated with 2 paediatric doses of a 7-valent pneumoccocal conjugate vaccine (*Prevnar*™, Wyeth Lederle Vaccines, USA) administered at the first and third vaccinations and one dose of *Haemophilus influenzae type b* vaccine (*Hiberix*™, GSK Biologicals, Belgium) at the second vaccination. Vaccines were administered at the Manhiça and Ilha Josina health centres.

Children were enrolled into two cohorts to measure the vaccine efficacy against either clinical malaria or malaria infection. In Cohort 1, based in Manhiça and Maragra, 1605 participants were followed-up using passive surveillance to detect clinical episodes of malaria. In Cohort 2, based in Ilha Josina, where malaria transmission intensity was 10 times higher [17], 417 participants were followed-up using active surveillance to detect malaria infection, through visits that started 14 days after the third vaccine dose and were done every 2 weeks for 2.5 months and then monthly for 2 additional months. In children from Cohort 2, asymptomatic parasitaemia was presumptively cleared with a combination of amodiaquine and sulfadoxine-pyrimethamine 14 days prior to dose 3.

Blood samples for determining anti-CSP antibody concentrations in both cohorts and anti-HBsAg antibodies (only Cohort 2) were obtained at study months 0 (prevaccination), 3 (1 month after the third vaccine dose), 8½, 21, 33 and 45. RF1-like antibodies were measured prior to vaccination and at study month 3, only in Cohort 2. Serum was separated for antibody determinations. Indirect fluorescent antibody tests (IFAT) for blood stage anti-parasite antibodies were performed prior to vaccination. Primary analysis of immunogenicity was performed on the ATP cohort (primary analysis).

2.3. Antibody responses to the RTS,S/AS02$_A$ vaccine

The levels of IgG antibodies to the NANP repeat region of CSP (B cell epitope) were measured by a standard, validated enzyme-linked immunosorbent assay (ELISA) using plates adsorbed with the R32LR antigen at a GSK validated laboratory (CEVAC, University of Ghent, Belgium). Antibody concentrations were calculated using a reference standard curve with a 4 parameter logistic fitting algorithm and expressed in EU/mL, with cut-off set at 0.5 EU/mL [18].

Anti-HBsAg antibody levels were measured only in samples from Cohort 2 at GSK laboratories by ELISA with a commercial kit (AUSAB EIA, Abbott Laboratories, Abbott Park, IL) for the first 5 samplings, and with an in-house developed HBsAg ELISA for the last month 45 sample (described elsewhere) [19]. RF1-like antibodies levels were determined using an in-house developed ELISA based competition assay with plate adsorbed HBs antigen, performed at CEVAC, University of Ghent, Belgium. Dilutions of the test samples and the reference serum were mixed with a fixed amount of RF1 mAb that was revealed through a colorimetric reaction. The signal obtained was inversely proportional to the amount of anti-RF1 like antibodies present in the samples. The amount of antibody competing with RF1 mAb for binding to the coated HBsAg was quantified by comparison to a reference serum using a 4 parameters equation (Softmax Pro Software), with an assay cut-off of 33 EU/ml.

2.4. Hepatitis B virus surface antigen

HBsAg levels were determined in both cohorts by ELISA with a commercial kit (ETI-MAK-4® DiaSorin®, Saluggia, Italy) at the Microbiology Service of Hospital Clinic, Universitat de Barcelona, Spain, according to the manufacturer's instructions.

2.5. Antibodies to blood-stage P. falciparum antigens by IFAT

To determine the level of naturally acquired *P. falciparum*-specific antibodies prior to vaccination, IFAT in baseline serum samples from children in the two study cohorts was conducted at the Barcelona Center for International Health Research (CRESIB, Hospital Clinic, Universitat de Barcelona, Spain). *In vitro* cultures containing mostly mature asexual blood stages of *P. falciparum* strains were grown at GSK Tres Cantos, Madrid, Spain. A pool was

prepared with a mixture of 3D7, K1, FCR3 and HB3 cultures, and parasitized erythrocytes were harvested and washed twice in PBS. Cells were resuspended to 3–5% hematocrit in PBS and 20–25 μl aliquots were placed onto 12-well multispot slides (Cell-Line Associates, Newfield, NJ, USA), dried, packed and stored at −20 °C in self-sealed plastic bags containing silica gel as desiccant.

Two-fold serial dilutions of the test sera were prepared (highest dilution tested was 1/81,920), and 25 μl of each serum dilution together with positive and negative control sera were placed onto acetone-fixed IFAT slides containing whole *P. falciparum* parasites and incubated in a wet chamber for 1 h at room temperature. After washing the slides 3 times with PBS-Tween 0.05%, 15 μl of FITC-labelled secondary antibody diluted in Evans Blue (1/100) were added and incubated for 30 min at 37 °C. Slides were washed 3 times with PBS-Tween 0.5%, mounted with buffered glycerine containing DAPI (1:100,000), and examined with a NIKON fluorescence microscope. The highest dilution giving positive fluorescence above the negative control levels was scored under the UV light. For each reading and each antibody, the end-point titer corresponded to the reciprocal of the greatest serum dilution that yielded a positive fluorescence.

The protocols (NCT00197041 and NCT00323622) were approved by the Mozambican National Bioethics Committee, the Hospital Clinic of Barcelona Ethics Review Committee and the PATH Human Subjects Protection Committee and implemented according to the International Conference of Harmonization and Good Clinical Practices guidelines. A Local Safety Monitor and a Data and Safety Monitoring Board oversaw the design, conduct and results of the trial.

2.6. Statistical analysis

For each treatment group, the seropositivity (S+) rate for anti-CSP antibodies (proportion of subjects with anti-CSP antibody concentration of ≥0.5 EU/mL) and their 95% Confidence Intervals (CI) were tabulated for each time point. Reverse cumulative distribution curves [20] were plotted stratified by age at day 0 (<24 months, ≥24 months) for serum antibody titers measured prior to immunization and at months $8^{1/2}$, 21 and 45.

For each treatment group in Cohort 2, the seroprotection (SP) rate for anti-HBsAg antibodies (proportion of subjects with anti-HBsAg antibody titers of ≥10 mIU/mL) and their 95% CI were tabulated for each time point. GMTs for anti-HBsAg antibodies measured in mIU/mL with 95% CI were calculated for each group at each time point when a serology sample was taken.

The seroconversion rate for anti-RF1 antibodies (proportion of subjects with anti-RF1 antibody titers of ≥33 mIU/mL) were tabulated with 95% CI for all time points at which anti-RF1 antibodies were measured.

GMT calculations were performed by taking the anti-log of the mean of the log titer transformations (log base 10). Titers below the cut-off were assigned an arbitrary value of half the cut-off of the assay for the purpose of GMT calculation.

The relationship between blood stage IFAT titers and anti-CSP antibodies in children vaccinated with RTS,S/AS02$_A$ was assessed by multiple regression methods. Age at vaccination was categorized in four groups, each one corresponding to a one year interval.

The relation between anti-CSP antibody concentrations as measured 30 days post dose 3 and the risk of infection and clinical malaria was assessed in RTS,S/AS02$_A$ recipients. The hazard ratio of participants with anti-CSP antibody titers in the highest tertile against those in the lowest tertile was estimated, as well as the hazard ratio per ten-fold increase in the value of anti-CSP antibodies, using Cox regression models.

Table 1
Association between anti-CSP antibody responses and baseline IFAT titers, age and cohort in vaccinated children.

Variable	Relative change[a]	95% CI	*p* value
IFAT at baseline[b]	1.07	1.014–1.117	<0.0001
Cohort 1	1		0.04
Cohort 2	1.23	1.000–2.404	
1 year at D1	1	1	<0.0001
2 years at D1	0.64	0.0661–1.632	
3 years at D1	0.41	0.378–0.963	
4 years at D1	0.46	0.510–1.303	

Cohorts: 1 = Manhica, 2 = Ilha Josina; D1 = Dose 1; Global $p < 0.0001$.

[a] Relative change in anti-CSP antibody geometric mean concentration at 1 month post dose 3.

[b] Per doubling the value of IFAT.

Analyses were done with SAS version 8 (Cary, NC, USA) and STATA version 9.0 (College Station, TX, USA).

3. Results

A total of 2022 children aged 1–4 years were randomised and received at least one vaccine dose of RTS,S/AS02$_A$ or control vaccine. Of these, 1565 were included in the immunogenicity analysis: 795 in the RTS,S/AS02$_A$ group and 770 in the control group. Fig. 1 shows the trial profile for the study.

3.1. Anti-CSP antibody responses

The magnitude and longevity of anti-CSP antibodies in the two groups receiving RTS,S/AS02$_A$ (cohorts 1 and 2) as well as in the corresponding control groups is shown in Fig. 2. Among RTS,S/AS02$_A$ recipients, a robust response in the development of anti-CSP IgG is followed by a decay in antibody concentrations over the first 6 months of follow-up to about 25% of the initial level. However 42 months after dose 3, 96% of participants remained seropositive (30-fold higher compared to controls). Also worth noting is the apparent lack of increase in anti-CSP antibodies or the proportion of seropositives among the control group while being exposed to high *P. falciparum* transmission.

The effect of age at time of vaccination on antibody responses has been explored by plotting the reverse cumulative distribution curves for anti-CSP antibody GMTs by age group (<24 months, ≥24 months) in both cohorts at different follow up periods (Fig. 3). There is some evidence of higher immunogenicity 30 days after the third dose among the younger age group, but this difference disappears over the subsequent follow-up period.

We also explored the relationship between blood stage IFAT titers, as a reflection of intensity of *P. falciparum* transmission, and anti-CSP immunogenicity. IFAT baseline values were significantly higher in Cohort 2 (GMT [95% CI], 25,623 [21,360–30,737] in controls and 27,496 [22,520–33,571] in vaccinated) compared to Cohort 1 (2490 [2084–2976] in controls and 2449 [2107–2964] in vaccinated) reflecting the higher malaria transmission intensity in Ilha Josina. When doubling the IFAT titers at baseline, the vaccine-induced anti-CSP antibodies modestly increased by 1.07 (95% CI: 1.04–1.09, $p < 0.0001$) having adjusted for the effect of age and cohort. However, children in Cohort 2 had 1.23 (95% CI: 1.01–1.50, $p = 0.04$) times higher anti-CSP antibody titers compared to those in Cohort 1, having adjusted for both IFAT and age (Table 1).

We also looked at whether anti-CSP antibody responses induced by the RTS,S/AS02$_A$ were influenced by pre-vaccination HBsAg status (Table 2). In the RTS,S/AS02$_A$ group, 16 subjects in Cohort 1 and 9 in Cohort 2 were HBsAg positive at pre-vaccination. Responses to anti-CSP were slightly lower in HBsAg positive children in comparison to HBsAg negative participants. However, in both cohorts,

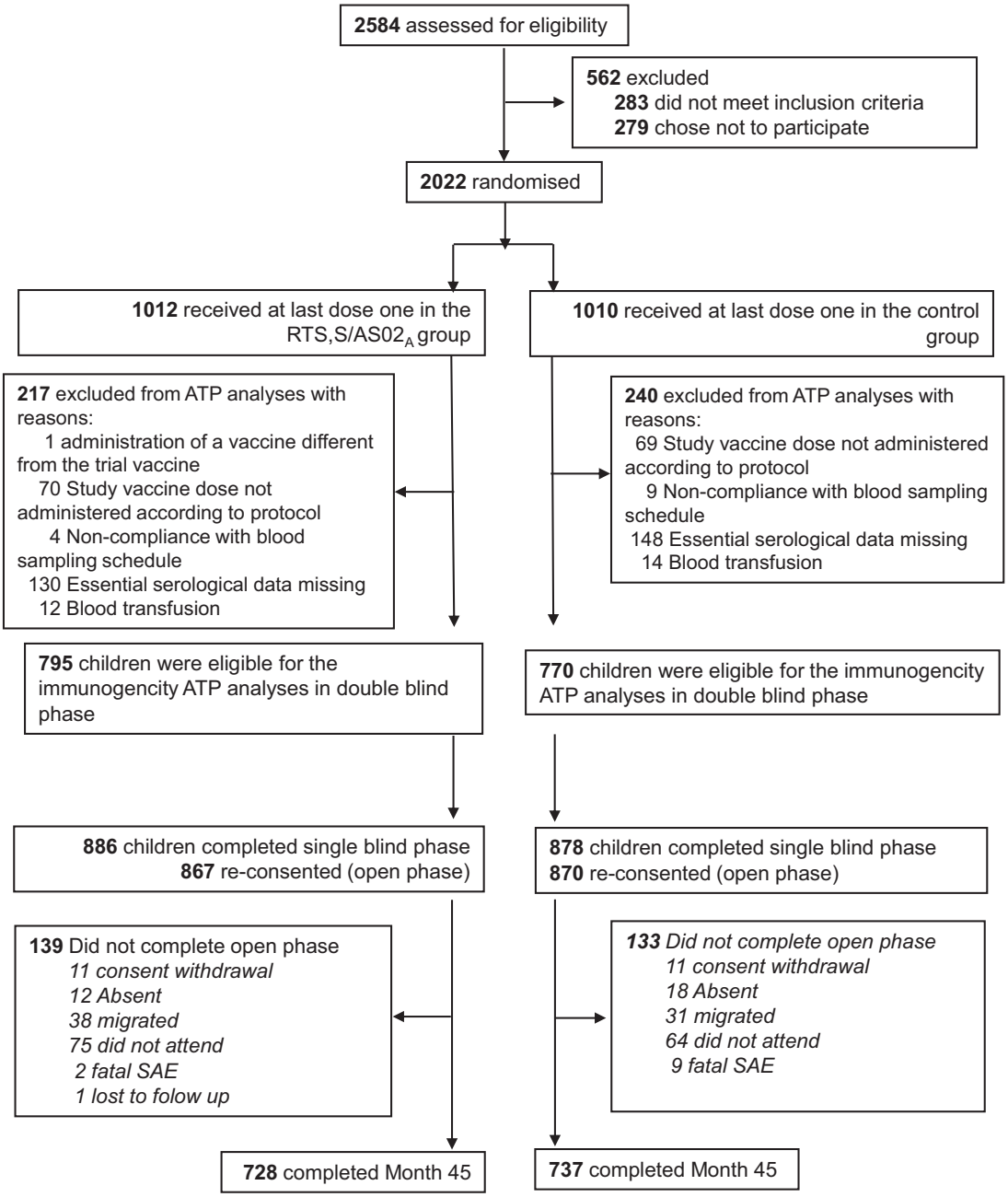

Fig. 1. Trial profile.

almost 100% of the subjects were seropositive for anti-CSP antibodies 1 month post dose 3.

Finally, we examined the relation between anti-CSP pre-vaccination antibodies and anti-HBsAg IgG titers. Subgroup analysis for Cohort 2 children showed no association between baseline anti-HBsAg antibody titers and anti-CSP IgG titers 1 month post dose 3. Even when adjusting by baseline IFAT and age, there was no evidence of increased RTS,S immunogenicity with higher anti-HBsAg titers (doubling the anti-HBsAg titers was associated with an increase of 1.01 (95% CI: 0.94–1.08) in the anti-CSP titers ($p = 0.854$)).

3.2. Anti-HBsAg antibody responses

Table 3 shows anti-HBsAg seroprotection rates and antibody GMTs, measured in samples from children in Cohort 2. We sub-divided age groups in two categories: a first group of children aged 24 months or older and that had not been previously immunised with hepatitis B vaccine, and a second group of participants younger than 2 years that had received hepatitis B vaccine through the routine EPI program. In children aged ≥24 months the seroprotective levels of anti-HBsAg antibodies at day 0 were approximately 20%, reflecting natural exposure. Immunisation with RTS,S/AS02$_A$ resulted in an increase of anti-HBsAg antibody titers from a GMT of 9.1 mIU/mL at baseline to 11368.6 mIU/mL 1 month post dose 3, and subsequently decreasing by 60% at month 8½ (4556 mIU/mL) and by 86% at month 45 (1557 mIU/mL). However, approximately 98% of subjects had seroprotective levels of anti-HBsAg antibodies at all time points. Among the control group that received *Engerix-B*™ approximately 90% of recipients were seroprotected following vaccination and remained so throughout the follow up. However, anti-HBsAg antibody titers were lower than among RTS,S recipients.

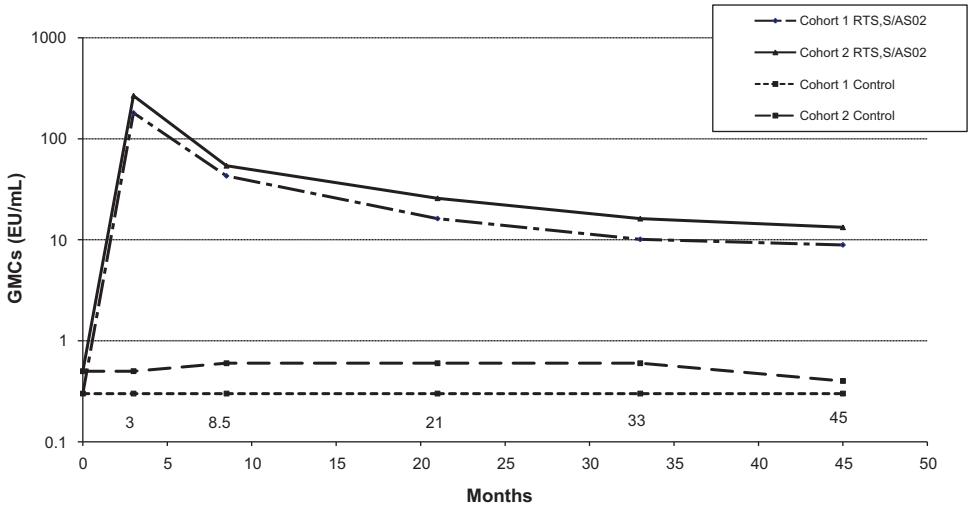

Fig. 2. The anti-CSP Antibody responses in children aged 1–4 years during the 45 months follow-up period. The figure represents the geometric mean concentration in the RTS,S/AS02$_A$ and control groups in both cohorts.

In children <24 months of age, the pre-vaccination seroprotective levels of anti-HBsAg antibodies were high in both the RTS,S/AS02$_A$ and control groups (>77%), reflecting the prior HBV immunisation. Following administration of RTS,S, seroprotection rates increased to 97% and remained so throughout the entire follow-up. GMT values for anti-HBsAg antibodies in this group increased from 62.9 mIU/mL at baseline to 51,035 mIU/mL 1 month post dose 3. This value decreased by 75% (to 13,642 mIU/mL) at month 8½ and by 93% (to 3324 mIU/mL) at month 45. Among the control group that received *Prevnar*™ and *Hiberix*™, seroprotection levels declined from an average of 79% pre vaccination to 56% at month 45. GMT of anti-HB antibodies also declined to 26.6 (95% CI 13.0–54.1) at the end of follow-up, reflecting the natural decay in antibodies and protection afforded by hepatitis B immunisation during the first year of life.

3.3. RF1-like antibody responses

Table 4 shows anti-RF1 antibody GMTs and seropositivity rates in Cohort 2 children at day 0 and 1 month after dose 3. No increase

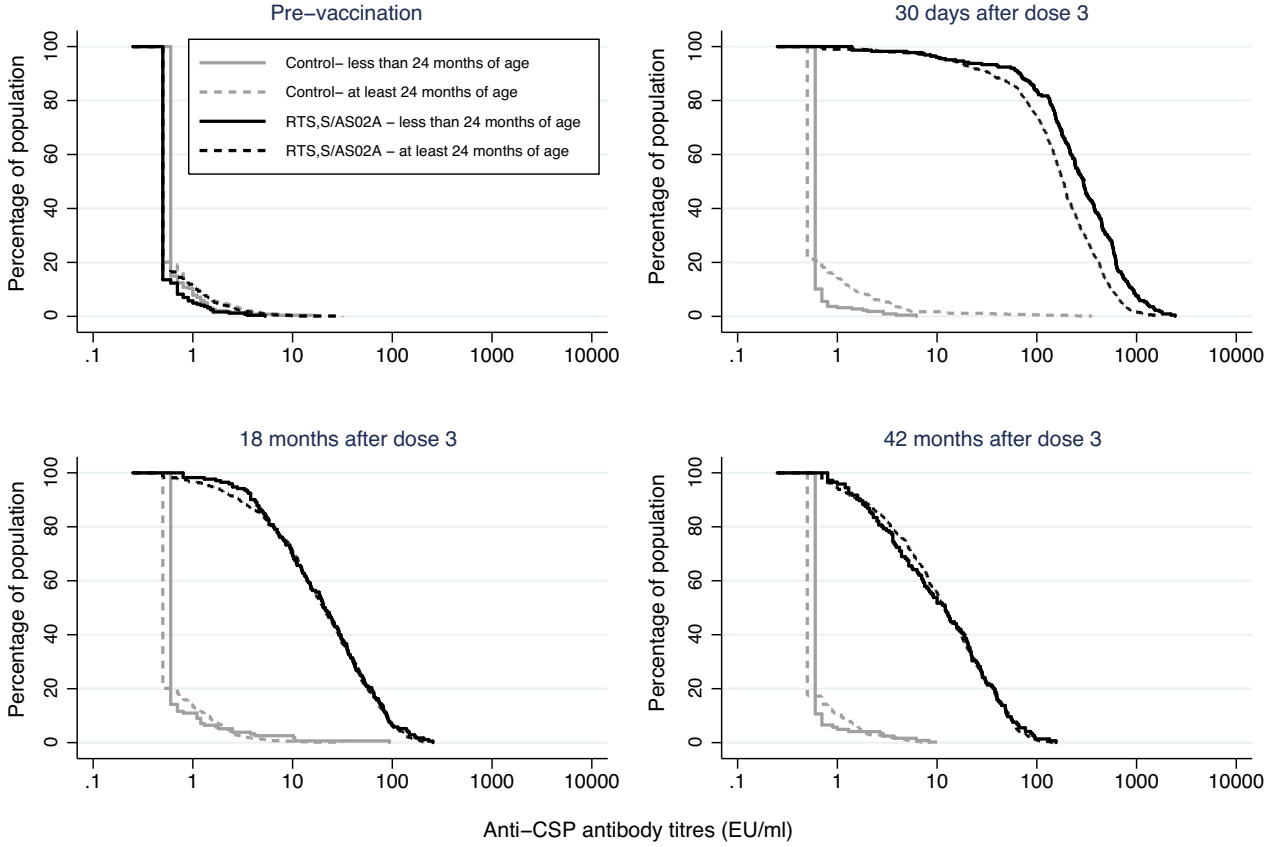

Fig. 3. Reverse cumulative distribution curves for the anti-CSP antibody titers by group, stratified by age at first dose in Cohort 1 and 2.

Table 2
Anti-CSP seropositivity rates and antibody GMCs by pre-vaccination HBsAg serostatus and study cohort in the RTS,S/AS02$_A$ vaccine group.

Cohort	Pre-vaccination status	Timing	*N*	Seropositivity for anti-CSP *n* (%)	Anti-CSP GMCs (95% CI)
	HBsAg	Baseline	584	69 (11.8)	0.3 (0.3–0.3)
	Negative	M3	584	579 (99.1)	181.4 (164.7–199.8)
		M8½	584	583 (99.8)	43.0 (38.8–47.7)
		M21	544	534 (98.2)	16.2 (14.4–18.1)
		M33	476	457 (96.0)	10.0 (8.8–11.4)
Cohort 1		M45	440	422 (95.9)	8.9 (7.8–10.1)
	HBsAg	Baseline	16	1 (6.3)	0.3 (0.2–0.3)
	Positive	M3	16	16 (100)	154.2 (89.4–265.9)
		M8½	16	16 (100)	42.0 (23.5–75.1)
		M21	14	14 (100)	17.7 (9.3–33.6)
		M33	13	13 (100)	11.4 (5.4–24.1)
		M45	11	11 (100)	9.5 (4.1–22.0)
	HBsAg	Baseline	176	62 (35.2)	0.5 (0.4–0.5)
	Negative	M3	169	169 (100)	267.9 (229.4–312.9)
		M8½	143	143 (100)	55.2 (45.5–67.0)
		M21	153	153 (100)	26.3 (22.0–31.5)
		M33	141	139 (98.6)	16.9 (13.7–20.9)
Cohort 2		M45	141	141 (100)	15.8 (13.0–19.2)
	HBsAg	Baseline	9	2 (22.2)	0.3 (0.2–0.5)
	Positive	M3	9	9 (100)	197.2 (88.7–438.3)
		M8½	9	9 (100)	30.1 (10.2–89.1)
		M21	9	9 (100)	18.2 (7.3–45.2)
		M33	8	7 (87.5)	7.7 (1.7–35.7)
		M45	9	9 (100)	10.0 (3.5–28.3)

N = number of subjects with available results.
N/% = number/percentage of subjects with titer within the specified range.
95% CI = 95% confidence interval.
M3, M8, M21, M33, M45 = months 3, 8, 21, 33 and 45 post dose 3
GMC = geometric mean concentrations.

in anti-RF1 seropositivity was observed in the *Prevnar*™ and *Hiberix*™ control group. Among participants receiving *Engerix-B*™, there was a 2.5 fold increase in anti-RF1 GMT and about 47% were seroprotected. However, among RTS,S/AS02$_A$ recipients seropositivity rates were greater than 98% in both age groups. There was also a marked increase in anti-RF1 GMT, being highest in children less than 24 months than in the older age group (a 55 and 22-fold increase in anti-RF1 antibody GMTs, respectively).

4. Discussion

Previous studies with RTS,S/AS02$_A$ in a endemic area of Mozambique showed that vaccination of children aged 1–4 years induces partial protection against infection and clinical malaria including severe disease [5,6,13], and the clinical benefit conferred by the vaccine is sustained over at least 45 months [13]. Here we report the immunogenicity of RTS,S/AS02$_A$ with respect to both the

Table 3
Anti-HBsAg seroprotection rates and antibody GMTs by age category and vaccine group in Cohort 2.

Age	Group	Timing	*N*	Seroprotection *n* (%)	Anti-HBs GMTs (95% CI)
	RTS,S/AS02$_A$	Baseline	44	34 (77.3)	62.9 (37.5–105.4)
		M3	41	40 (97.6)	51,035.4 (27,918.9–93,291.8)
		M 81/2	33	32 (97.0)	13,642.0 (7342.2–25,347.1)
		M21	37	36 (97.3)	5935.3 (3218.6–10,945.1)
		M33	33	32 (97.0)	4008.6 (2266.7–7089.1)
Less than 24 months at		M45	35	34 (97.1)	3323.8 (1908.4–5788.8)
day 0	*Prevnar*™ and	Baseline	42	33(78.6)	92.4 (47.1–181.1)
	Hiberix™	M3	33	26 (78.6)	67.7 (33.9–135.4)
		M81/2	31	23 (74.2)	40.1 (21.1–76.4)
		M21	37	23 (62.2)	35.5 (17.7–71.2)
		M33	33	16 (47.1)	20.3 (10.9–54.1)
		M45	35	18 (56.3)	26.6 (13.0–54.1)
	RTS,S/AS02$_A$	Baseline	148	28 (18.9)	9.1 (7.3–11.4)
		M3	134	132 (98.5)	11,368.6 (8518.9–15,171.6)
		M81/2	121	118 (97.5)	4556.4 (3499.8–5932.1)
		M21	125	123(98.4)	2877.0 (2241.5–3692.8)
At least 24 months old		M33	116	114 (98.3)	1842.5 (1413.7–2401.3)
at day 0		M45	115	113 (98.3)	1557.0 (1187.6–2041.4)
	Engerix-B™	Baseline	142	29 (20.4)	9.0 (7.2–11.2)
		M3	118	108 (91.5)	349.9 (236.7–517.0)
		M81/2	115	103 (89.6)	153.5 (110.6–213.0)
		M21	127	109 (85.8)	103.8 (74.8–144.1)
		M33	115	90 (78.3)	67.4 (47.5–95.7)
		M45	113	102 (90.3)	99.4 (73.1–135.1)

N = number of subjects with available results.
N/% = number/percentage of subjects with titer within the specified range.
95% CI = 95% confidence interval.
M3, M8, M21, M33, M45 = months 3, 8, 21, 33 and 45 post dose 3
GMT = geometric mean titer.

Table 4
Anti-RF1 seropositivity rates and antibody geometric mean titers (GMT) by age category in Cohort 2.

Age	Group	Timing	*N*	Seroprotection *n* (%)	Anti-RF1 GMT (95% CI)
Less than 24 months old at day 0	RTS,S/AS02$_A$	Baseline	43	2 (4.7)	20.2 (14.6–28.0)
		M3	39	39 (100)	1113.6 (810.2–1530.7)
	Prevnar™ and *Hiberix*™	Baseline	40	1 (2.5)	17.0 (16.0–18.1)
		M3	34	1 (2.9)	17.5 (15.5–19.7)
At least 24 months old at day 0	RTS,S/AS02$_A$	Baseline	146	4 (2.7)	19.0 (16.3–22.0)
		M3	132	130 (98.5)	421.7 (346.1–513.8)
	Engerix-B™	Baseline	143	2 (1.4)	17.9 (15.9–20.1)
		M3	107	50 (46.7)	43.2 (33.0–56.5)

N = number of subjects with available results.
N/% = number/percentage of subjects with titer within the specified range.
95% CI = 95% confidence interval.
M3 = month 3 post dose 3.
GMT = geometric mean titers.

P. falciparum and HBV components of the vaccine in the largest phase IIb trial of RTS,S/AS02$_A$ conducted and over a total surveillance period of 45 months. The RTS,S/AS02$_A$ candidate vaccine was shown to be immunogenic in young African children, inducing high anti-CSP antibody levels after three doses.

Recent Phase IIb trials of RTS,S/AS01$_E$ (a slightly modified Adjuvant System improving immunogenicity) in children have indicated that higher titers of anti-CSP IgG antibodies may be induced in children who have received previous immunization with HBV vaccine [21,22]. Several possible mechanisms have been proposed to explain this observation, including both B and/or T cell priming by prior HBV vaccination [21]. However, in our study we found no evidence that pre-vaccination anti-HBsAg antibody titers had an influence on the levels of anti-CSP antibodies induced by RTS,S/AS02$_A$.

High levels of antibodies to asexual blood-stage antigens acquired by natural exposure to *P. falciparum* infection were seen by IFAT, especially in Ilha Josina. IFAT served as an indirect measure to compare intensity of malaria transmission in the two trial sites [6]. RTS,S/AS02$_A$ recipients in Ilha Josina (Cohort 2) had higher anti-CSP antibodies throughout the follow-up period than those in Manhiça (Cohort 1). Together with the observed higher level of anti-CSP antibodies among the control group in Cohort 2 versus Cohort 1, and the higher production of anti-CSP antibodies following immunization in Cohort 2 compared to Cohort 1, it would appear that in areas of higher transmission, immunogenicity is higher and this may be a reflection of limited natural boosting.

Natural exposure induces a poor anti-CSP antibody response, including that achieved at high transmission. As such, pre-vaccination anti-CSP antibody levels were low in all study children and remained so in the control group of Cohort 2 throughout follow-up, therefore suggesting that parasite exposure induced poor anti-CSP responses (Fig. 2) that did not increase during the four year surveillance period despite a very high incidence of infection and disease; consequently we did not observe evidence of natural boosting of anti-CSP antibodies.

In the RTS,S/AS02$_A$ group, anti-CSP antibody levels peaked 30 days post dose 3, declining over the next 6 months to about ¼ of the peak level, but remaining 30-fold higher than the cut-off level until month 45 compared to pre-vaccination GMT or control individuals. These results are consistent with those observed in a 5 year follow-up study conducted in Gambian semi-immune adults vaccinated with RTS,S/AS02$_A$ [23] It is not clear if the sustained IgG levels, presumably by long-lived plasma cells in the bone marrow, are sufficient for an antibody-mediated contribution in protection. It would be of interest to study the kinetics of memory B-cell response to CSP stimulus and the magnitude of expansion and up-regulation of antibody production following subsequent exposures to *P. falciparum* sporozoite infections. Alternatively, vaccine-specific antibodies could last for decades in the absence of antigenic re-exposure, as has been shown with smallpox vaccination [26] and recently with other malaria antigens upon natural infection [24,25]. Other human vaccines (e.g. live measles, mumps, and poliovirus) also induce serum antibody responses that persist for years, however it is unclear how durable antibody responses may be affected by intermittent re-exposure to the antigen, and this question remains open also for RTS,S/AS02$_A$ vaccination.

Another interesting observation is the effect of age on the antibody titers induced by the vaccine. One month after dose 3 of RTS,S/AS02$_A$, anti-CSP IgG levels were higher in the younger children (<2 years) compared to the older children (≥2 years), further demonstrating that the vaccine is highly immunogenic also in young children.

We previously assessed the role of anti-CSP antibody responses induced by RTS,S/AS02$_A$ in protection against malaria and found that higher levels of anti-CSP IgG were associated with a lower risk of infection in Cohort 2 [26]. The hazard ratio for *P. falciparum* infection of children per ten-fold increase in the value of anti-CSP antibodies was 0.41 (95% CI 0.28–0.60, $p < 0.001$). This is consistent with results from subsequent infant phase I/IIb trials of RTS,S/AS02$_D$ conducted in the same area and in Tanzania, in which high antibody titers were also associated with protection against infection [7,8]. However, no association was found between peak CSP antibody responses and protection against clinical malaria in any of the cohorts.

This indicates that anti-CSP antibodies, probably together with other cellular immune responses, as shown in other RTS,S/AS studies in naïve adults [11] and as measured in Mozambican infants [9], are involved in initial protection against infection. However, higher anti-CSP antibody titers did not predict greater protection against clinical malaria [13]. One hypothesis is that the longevity of protection found in Cohort 1 may be explained by the fact that as a "leaky" vaccine, RTS,S/AS vaccination in the context of ongoing malaria exposure has allowed the acquisition of natural immunity to liver or blood-stage antigens in vaccine recipients that are similar to those that develop in unvaccinated controls [26]. Additional studies will be required to determine the validity of this hypothesis. In addition to the magnitude of the antibodies, the quality and type of the IgGs may be relevant to their anti-parasite effector role. Therefore, for a more complete understanding of the mechanism of action of RTS,S/AS, future studies should include evaluation of IgG isotypes, avidity and affinity and the biological function, e.g. the neutralizing effect of antibodies to inhibit the migration, invasion or development of sporozoites or an opsonizing capacity [27] that may facilitate the phagocytosis and digestion of sporozoites by antigen presenting cells. Likewise, the mechanism of induction and maintenance of memory B cell responses to RTS,S/AS needs to be investigated more thoroughly to better understand the pattern of affinity maturation and kinetics of vaccine-specific anti-CSP

antibody responses. Similarly, the contribution of cellular immunity to RTS,S/AS-induced protection needs further investigation. It remains to be established whether a fourth vaccine dose will boost the antibody responses and help sustain their durability. This will be tested in the ongoing phase III trial of RTS,S/AS01$_E$, the primary objective of which is to gather the safety, efficacy and immunogenicity data necessary for vaccine registration. The study will also provide a unique opportunity to try to understand the vaccine mechanisms of action and investigate immune correlates of vaccine-induced protection. Possibilities for improving on the partial protection against malaria observed in previous RTS,S/AS0 trials would include the combination of this vaccine with other *P. falciparum* antigens and/or delivery systems in heterologous prime-boost immunization schemes.

Finally, we evaluated the antibody responses induced by the HBV portion of RTS,S. This trial gave us the unique opportunity of having a head to head comparison of RTS,S and a licensed hepatitis B vaccine (*Engerix-B*™) in children older than 24 months. RTS,S induced higher antibody levels and achieved greater levels of seroprotection than did the commercially available hepatitis B vaccine.

5. Conclusions

RTS,S/AS02A has been found to be safe and to induce moderate levels of protection against different malaria endpoints. Further work is required to understand the exact immune mechanisms of action. Detectable anti-CSP GMCs antibodies persisted up to month 45 (42 months after dose 3) in RTS,S/AS02$_A$ recipients, and at least 97% of the vaccine recipients had seroprotective levels of anti-HBsAg antibodies 45 months after immunization. The anti-HBsAg antibody response was significantly higher with RTS,S/AS02$_A$ compared to *Engerix-B*™. The candidate malaria vaccine was highly immunogenic for anti-CSP and anti-HBsAg antibodies, especially in children younger than 24 months.

This vaccine, currently undergoing phase III trials will require further improvements to expand its protection. Understanding the immune mechanisms of action will increase our chance of improving its performance.

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